

Award Number: W81XWH-06-1-0737

TITLE: Erythropoietin and Breast Cancer

PRINCIPAL INVESTIGATOR: Arthur J. Sytkowski, MD

CONTRACTING ORGANIZATION:

Beth Israel Deaconess Medical Center
Boston, MA 02215-5400

REPORT DATE: March 2008

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01/03/2008		2. REPORT TYPE Final		3. DATES COVERED (From - To) Sep 1, 2006- Feb 27, 2008	
4. TITLE AND SUBTITLE Erythropoietin and Breast Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-06-1-0737	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Arthur J. Sytkowski, MD				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Beth Israel Deaconess Medical Center Boston, MA 02215-5400				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACTErythropoietin (Epo) is the prime regulator of red blood cell production, . principally by means of "anti-apoptotic" action. Epo also acts on other cells and tissues outside of the hematopoietic system, including endothelium, central nervous system, reproductive system and gut. Epo receptors (EpoR) have also been identified on breast cancer (CaB), and there is an <i>in vitro</i> study suggesting that these EpoR on CaB cell lines may be functional. The presence of functional EpoR on cancer cells is of concern since Epo is used to treat anemia associated with chemotherapy and to improve tumor oxygenation for radiotherapy. This concern was heightened when a trial of Epo in CaB patients was terminated early due to decreased survival in the Epo-treated group. Since CaB patients receive Epo therapy, an anti-apoptotic effect of Epo on CaB cells would have adverse consequences. It is of vital importance to determine the functionality of the EpoR on CaB cells <i>in vivo</i> . We screened CaB lines for EpoR expression and used siRNA technology to develop lines that had reduced EpoR. Reduction of EpoR resulted in a significant reduction in cell growth <i>in vitro</i> . These results may have profound implications for the magement of breast cancer patients					
15. SUBJECT TERMS Erythropoietin; receptor; siRNA; cell proliferation					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
UU	UU	UU	UU	12	19b. TELEPHONE NUMBER (include area code)

Table of Contents

Page

Introduction.....	4
Body.....	4
Key Research Accomplishments.....	9
Reportable Outcomes.....	10
Conclusion.....	11
References.....	11
Appendices.....	12

INTRODUCTION

Erythropoietin (Epo) is the prime regulator of red blood cell production. It increases red cell numbers principally by means of an “anti-apoptotic” action on erythroid progenitors. Importantly, Epo also acts on other cells and tissues outside of the hematopoietic system, including endothelium, central nervous system, reproductive system and gut (reviewed in [1]). Epo receptors (EpoR) have also been identified on breast cancer (CaB) and other malignant human cells, and there is an *in vitro* study suggesting that these EpoR on CaB cell lines may be functional [2, 3]. EpoR on prostate cancer cells have been shown definitively to be functional [4]. The presence of functional EpoR on cancer cells is of particular concern since recombinant Epo is used widely to correct the anemia associated with chemotherapy and to improve tumor oxygenation for radiotherapy. This concern was heightened recently when a trial of Epo in CaB patients was terminated early due to decreased survival in the Epo-treated group [5]. Given the large number of CaB patients who receive Epo therapy, an anti-apoptotic effect of Epo on CaB cells *in vivo* would have profound adverse consequences. Therefore, it is of vital importance to determine the functionality of the EpoR on CaB cells *in vivo*.

BODY

We obtained several CaB cell lines from the American Type Culture Collection. They included MCF7, MCF10A (a control normal mammary epithelial cell line), SKBR, MDA-MB-231, HS 578T and T47D. After trying several sets of primers to identify the EpoR by RT-PCR, we settled on a pair that gave reproducible results. As seen in **Figure 1**, each of the CaB lines expressed the EpoR. Notably, the non-malignant MCF10A control normal mammary epithelial cell line did not.

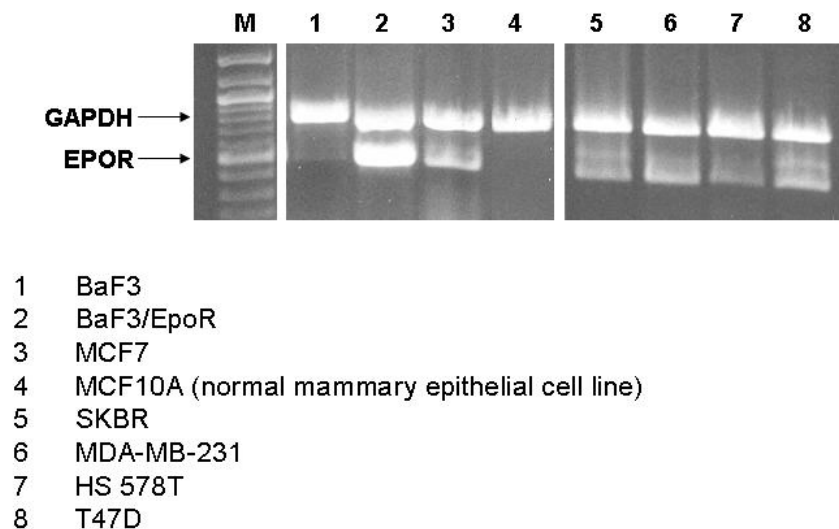


Figure 1. Expression of EpoR by human breast cancer cell lines demonstrated by RT-PCR. M, molecular weight markers; 1, BaF3 cells negative control; 2, BaF3/EpoR cells positive control; 3, 5-8, breast cancer cell lines; 4, normal human mammary epithelial cell line.

The amount of EpoR mRNA expressed by each of the CaB cell lines was approximately 1 % of that expressed by the positive control BaF3/EpoR cells [6]. Since BaF3/EpoR cells express approximately 3000 EpoR/cell on their surface (determined by us using radiolabeled Epo binding experiments), this would mean that CaB cells express about 30 EpoR/cell, below the limits of detection of our binding assay. However, we still attempted radiolabeled Epo binding experiments with the CaB lines (not shown). We were not able to detect specific binding to EpoR above the non-specific binding curve for all lines tested. We have obtained similar results when testing human ovarian and prostate cancer cell lines that we have shown, nevertheless, to express functional EpoR [4, 7, 8].

We tested the effect of short-term (48 hr) exposure to recombinant human Epo (rhEpo) on the growth of CaB cells in liquid culture **Figure 2**. We found no significant effect of short term rhEpo exposure on CaB growth at all concentrations tested. This result is identical to our published results on ovarian cancer [7, 8] but is in contrast to the significant growth effect that we observed on prostate cancer cells [4].

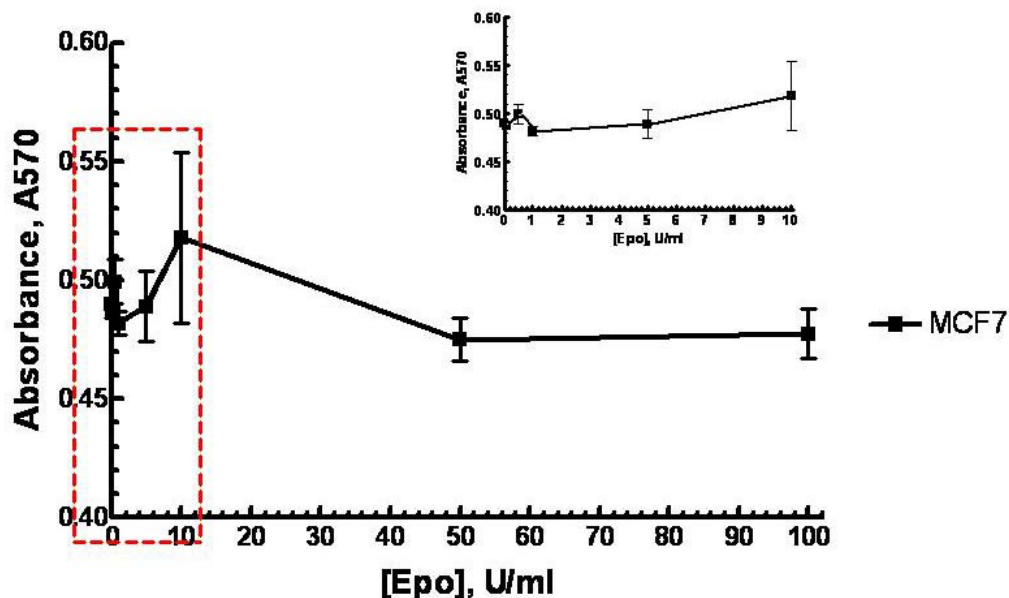


Figure 2. Short-term (48 hr) exposure of MCF7 cells to rhEpo has no significant effect on growth in liquid culture.

Though not proposed originally, we discovered that long-term (>30 days) exposure of CaB cell to rhEpo results in a change in phenotype characterized by an increased growth rate even in the absence of further add rhEpo. MCF7 cells were grown for 30 days in the presence of 5 U rhEpo/ml or 35 U rhEpo/ml, results in derivative cell lines designated MCF7/+5 and MCF7/+35, respectively. As seen in **Figure 3**, both MCF7/+5 and MCF7/+35 grew faster than did MCF7, with MCF7/+35 growing the fastest of the three.

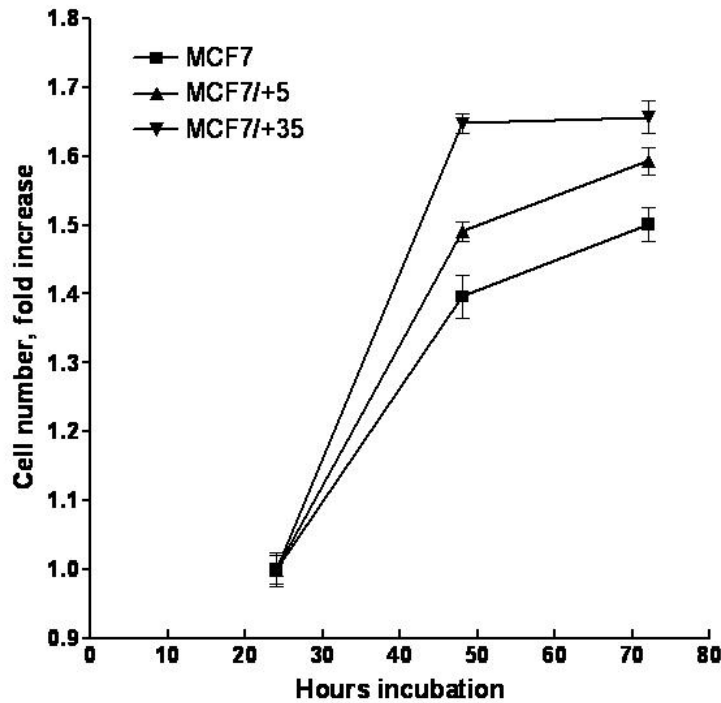


Figure 3. Long-term exposure of breast cancer cells to rhEpo results in an enhanced growth phenotype.

In contrast to the lack of short-term rhEpo effect on growth in liquid culture (see Figure 2), rhEpo did exhibit a modest but significant stimulatory effect on CaB cell colony formation in soft agar (**Figures 4 and 5**), even at concentrations readily achieved in patients treated with rhEpo for cancer-related anemia. This effect was equivalent to that observed using 10 nM β -estradiol, a known growth stimulant for many CaB cell lines.

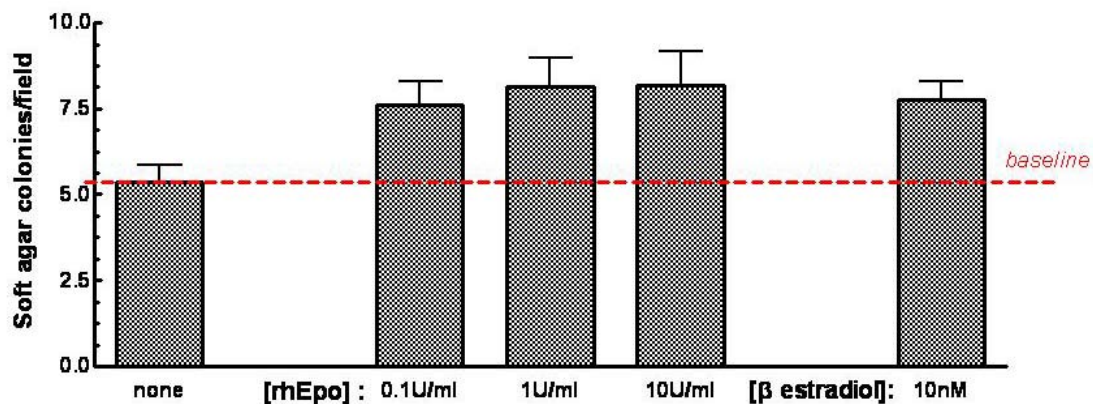


Figure 4. Treatment of breast cancer cells with rhEpo increases colony formation in soft agar.

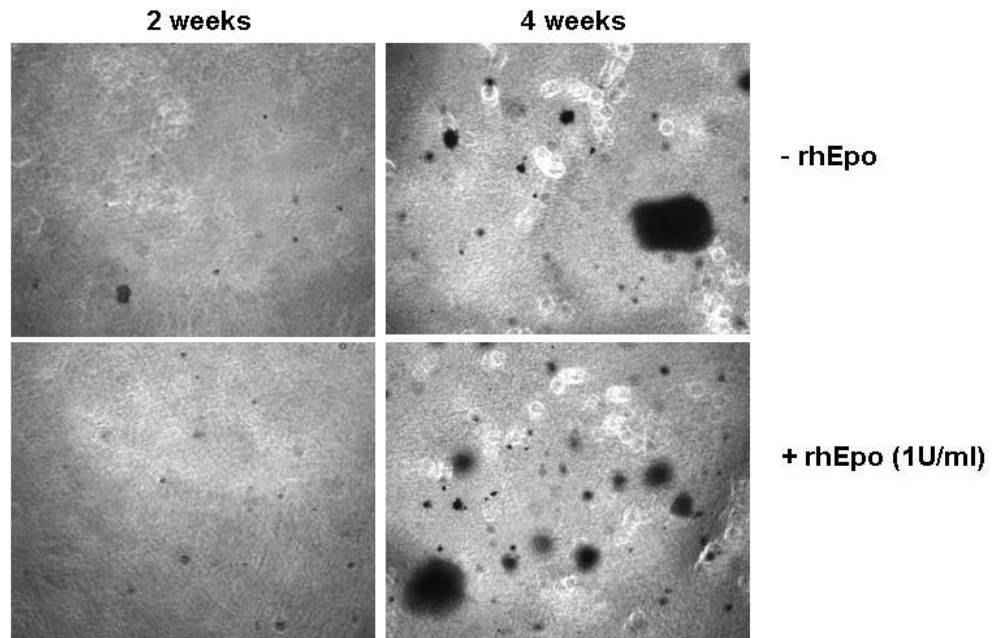


Figure 5. Effect of rhEpo treatment on MCF7 cell colony formation.

Importantly, long-term rhEpo-treated cells formed even more colonies in soft agar, even in the absence of added rhEpo, consistent with a change in growth phenotype caused by long-term rhEpo exposure (Figure 6).

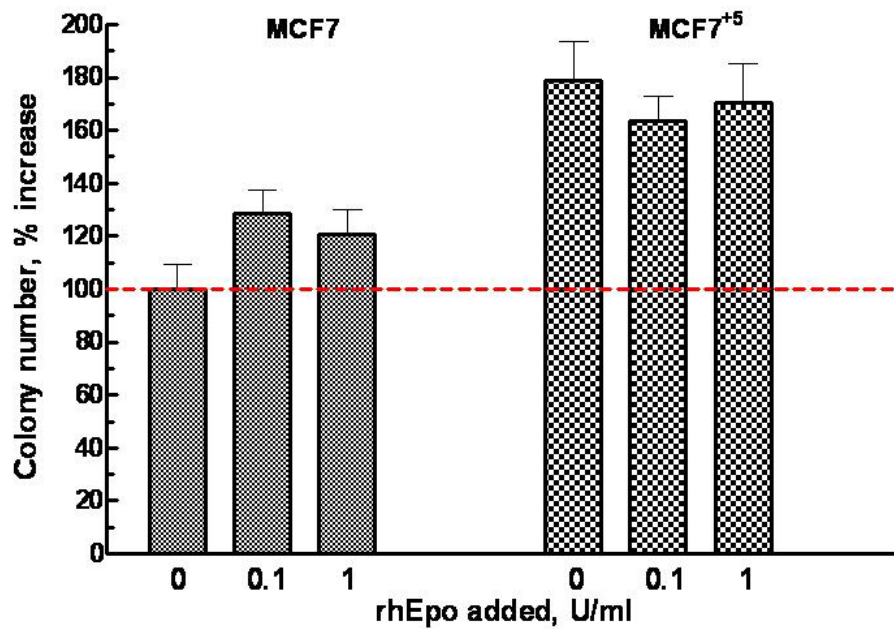


Figure 6. Long-term rhEpo treatment dramatically increases MCF7 colony formation.

In an effort to produce CaB cell lines with the EpoR down-regulated, we first attempted to use antisense RNA technology, with which we have considerable experience[9-11] . However, after many attempts over several months, we did not succeed in reliably down-regulating the EpoR using antisense. Toward the end of the award period we developed an siRNA method for down-regulating the EpoR in CaB cells. We first used transient transfection of MCF7 cells with an siRNA expression vector. As seen in **Figure 7**, semi-quantitative RT-PCR shows reduced expression of EpoR in the MCF7/EpoR-KD (knock-down) cells compared to MCF7 and MCF7 transfected with a non-targeting control vector. Quantitative real-time PCR confirmed a significant down-regulation (knock-down) of EpoR expression in the MCF7/EpoR-KD cells.

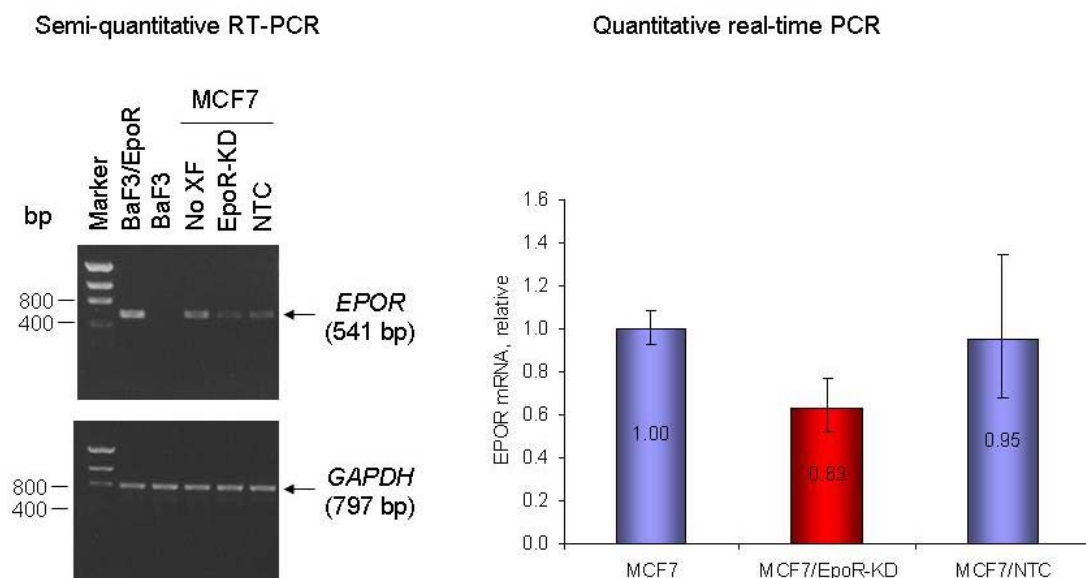


Figure 7. Quantification of EpoR expression in transient knock-down MCF7 cells.

Importantly, transient knock-down of EpoR leads to a reduced growth rate of the cells, even in the absence of added rhEpo (**Figure 8**).

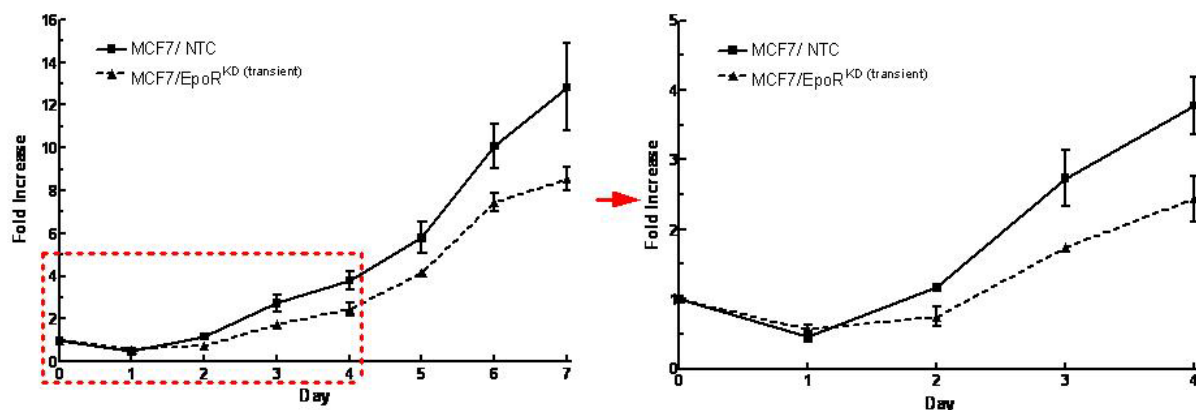


Figure 8. Transient Knock-down of EpoR leads to a slower growth rate of MCF7 cells.

We next carried out stable transfection of MCF7 cells. As shown in **Figure 9**, we succeeded in isolating two MCF7 clones with stably knocked-down EpoR expression. We plan to carry out the in vivo mouse experiments proposed once new funding is available.

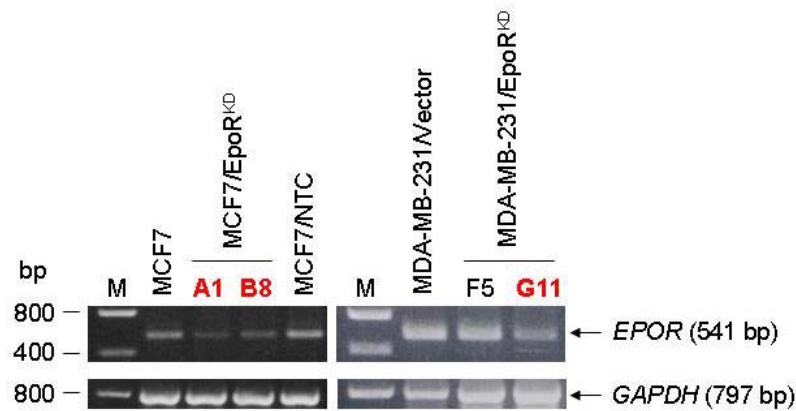


Figure 9. Quantification of EpoR expression in stable knock-down clones of MCF7 cells.

This concludes the progress made during the project period.

KEY RESEARCH ACCOMPLISHMENTS

- Identified EpoR expression in all Cab cell lines examined.
- Found no EpoR expression by non-malignant breast epithelial cell line MCF-10A
- Showed that added rhEpo stimulates colony formation in soft agar
- Discovered that long-term exposure to rhEpo changes the phenotype and increases growth rate and colony formation
- Succeeded in down-regulating (knocking-down) EpoR expression transiently using siRNA
- Showed that EpoR knock-down cells grew more slowly
- Developed clones of MCF7 with EpoR stably knocked-down

REPORTABLE OUTCOMES

Papers published:

Feldman L, Wang Y, Rhim JS, Bhattacharya N, Loda M, Sytkowski AJ. Erythropoietin Stimulates Growth and STAT5 Phosphorylation in Human Prostate Epithelial and Prostate Cancer Cells. *Prostate*. 2006; 66: 135-145.

Debeljak N, Feldman L, Davis KL, Komel R, Sytkowski AJ. Variability in the Immunodetection of His-tagged Recombinant Proteins. *Analytical Biochemistry* 2006; 359: 216-223.

Sytkowski, AJ. Does erythropoietin have a dark side? Epo signaling and cancer cells. *Science STKE* 2007, pe38.

Debeljak, N and Sytkowski, AJ. EpoR. UCSD-Nature Molecule Pages. 2007.
(doi:10.1038/mp.a000863.01)

Jeong J-Y, Feldman L, Solár P, Szenajch J, Sytkowski AJ. Characterization of Erythropoietin Receptor and Erythropoietin Expression and Function in Human Ovarian Cancer Cells. *International Journal of Cancer* 2008; 122(2): 274-280.

Solár P, Feldman L, Jeong J-Y, Busingye J, Sytkowski AJ. Erythropoietin Treatment of Human Ovarian Cancer Cells Results in Enhanced Signaling and a Paclitaxel-Resistant Phenotype. *International Journal of Cancer*. 2008; 122(2): 281-288.

Debeljak N and Sytkowski AJ. Erythropoietin: New Approaches to Improved Molecular Designs and Therapeutic Alternatives. *Current Pharmaceutical Design*, 2008; 14:1302-1310.

Jelkmann W, Bohlius J, Hallek M, Sytkowski AJ. The erythropoietin receptor in normal and cancer tissues. *Critical Reviews in Oncology/Hematology*, 2008; 67(1): 39-61.

Solár P, Koval J, Mikeš J, Kleban J, Solárová Z, Lazúr J, Hodorová I, Fedoročko P, Sytkowski AJ. Erythropoietin Inhibits Apoptosis Induced By Photodynamic Therapy In Ovarian Cancer Cells. *Molecular Cancer Therapeutics*, 2008; in press.

Abstract presentation:

Laurie Feldman, Jee-Yeong Jeong, Kerry L. Davis, Amanda L. Socha, Klemen Spaninger, Arthur J. Sytkowski; Role of the erythropoietin receptor in breast cancer pathobiology. Presented in poster form and orally at the Fifth Department of Defense (DOD) Breast Cancer Research Program (BCRP) Era of Hope 2008 Meeting, June 25–28, 2008, Baltimore, MD.

Cell lines:

MCF7 cell lines transfected with stable vectors expressing human EpoR siRNA.

CONCLUSIONS

The erythropoietin receptor EpoR is expressed by human breast cancer cells. These EpoR are functional and trigger a mitogenic response when activated by Epo. This has important clinical implications for breast cancer patients receiving erythropoietin.

REFERENCES

1. Sytkowski, A.J., *Erythropoietin: Blood, Brain and Beyond*. 2004, Weinheim: Wiley-VCH.
2. Acs, G., P.J. Zhang, T.R. Rebbeck, P. Acs, and A. Verma, *Immunohistochemical expression of erythropoietin and erythropoietin receptor in breast carcinoma*. *Cancer*, 2002. **95**(5): p. 969-81.
3. Acs, G., P. Acs, S.M. Beckwith, R.L. Pitts, E. Clements, K. Wong, and A. Verma, *Erythropoietin and erythropoietin receptor expression in human cancer*. *Cancer Research*, 2001. **61**(9): p. 3561-5.
4. Feldman, L., Y. Wang, J.S. Rhim, N. Bhattacharya, M. Loda, and A.J. Sytkowski, *Erythropoietin stimulates growth and STAT5 phosphorylation in human prostate epithelial and prostate cancer cells*. *Prostate*, 2006. **66**(2): p. 135-45.
5. Leyland-Jones, B., V. Semiglazov, M. Pawlicki, T. Pienkowski, S. Tjulandin, G. Manikhas, A. Makhson, A. Roth, D. Dodwell, J. Baselga, M. Biakhov, K. Valuckas, E. Voznyi, X. Liu, and E. Vercammen, *Maintaining Normal Hemoglobin Levels With Epoetin Alfa in Mainly Nonanemic Patients With Metastatic Breast Cancer Receiving First-Line Chemotherapy: A Survival Study*. *J Clin Oncol*, 2005. **23**(25): p. 5960-5972.
6. Showers, M.O., J.F. Moreau, D. Linnekin, B. Druker, and A.D. D'Andrea, *Activation of the erythropoietin receptor by the Friend spleen focus-forming virus gp55 glycoprotein induces constitutive protein tyrosine phosphorylation*. *Blood*, 1992. **80**(12): p. 3070-8.
7. Jeong, J.-Y., L. Feldman, P. Solar, J. Szenajch, and A.J. Sytkowski, *Characterization of erythropoietin receptor and erythropoietin expression and function in human ovarian cancer cells*. *International Journal of Cancer*, 2008. **122**(2): p. 274-280.
8. Solar, P., L. Feldman, J.-Y. Jeong, J.R. Busingye, and A.J. Sytkowski, *Erythropoietin treatment of human ovarian cancer cells results in enhanced signaling and a paclitaxel-resistant phenotype*. *International Journal of Cancer*, 2008. **122**(2): p. 281-288.
9. Chern, Y.J., C. O'Hara, and A.J. Sytkowski, *Induction of hemoglobin synthesis by downregulation of MYB protein with an antisense oligodeoxynucleotide*. *Blood*, 1991. **78**(4): p. 991-6.
10. Li, Y., K.L. Davis, and A.J. Sytkowski, *Protein kinase C-epsilon is necessary for erythropoietin's up-regulation of c-myc and for factor-dependent DNA synthesis. Evidence for discrete signals for growth and differentiation*. *Journal of Biological Chemistry*, 1996. **271**(43): p. 27025-30.
11. Cui, K., M. Coutts, J. Stahl, and A.J. Sytkowski, *Novel interaction between the transcription factor CHOP (GADD153) and the ribosomal protein FTE/S3a modulates erythropoiesis*. *Journal of Biological Chemistry*, 2000. **275**(11): p. 7591-6.

APPENDIX

Personnel Receiving Pay From the Research Effort

Arthur J. Sytkowski, MD